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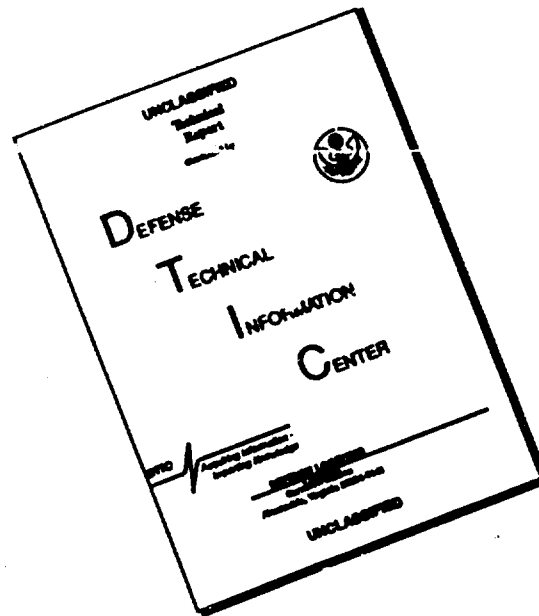
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SUBMICROSCOPIC CHANGES IN THE RAT LUNG AFTER  
ADMINISTRATION OF A CUMARIN DERIVATIVE

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Clinical and experimental investigations have revealed that coumarin preparations can be credited not only with action upon the blood coagulation system. Instead, they also increase the capillary permeability and can cause hemorrhages. Both of these phenomena can be explained only in the light of the changes occurring in the small blood vessels which -- as described in this communication for the case of an oxycoumarin derivative -- can be illustrated. The preparation used appeared particularly suitable in connection with preliminary experiments or electron microscope investigations because, if the experiment lasts sufficiently long, it leads rather constantly to hemorrhages in the lung parenchyma which are macroscopically quite easily recognizable.

Material and Methods

We took mostly female white rats, weighing 70-500 g and, upon suggestion of the producer, we added 3-(alpha-phenyl-beta-acetylhyl)-4-oxycoumarin ("Ratron substance, 80%, respectively, 87%" -- we want to thank the Delitia Delitzsch Company here for letting us have the quantities of the preparation used in this experimentation), in a 0.5% concentration to the fodder which was renewed daily. In a few of our experiments we did not administer this substance on individual days. Four more rats were given Ratron through a stomach probe. The experiment lasted between 2 hours and 8 days. In connection with the short experiment, the animals were starved 2 days prior to the first administration of the substance so that we could guarantee the greatest possible introduction of the preparation after feeding was resumed. Including the controls, we had a total of 56 rats in this experiment. Then we took 19 animals and administered local anesthesia with Jenacain, after a blow on the neck, or full narcosis by means of hexobarbital, respectively, ether, in order to remove hemorrhaged and adjoining

macroscopically imperceptible lung tissue for the electron microscope preparation. The tissue was fixed in an isotonic, buffered  $\text{OsO}_4$  solution and it was then embedded in methacrylate and, in some cases, also in Vestopal W; before that it had been contrasted, during dehydration, with phosphotungstic acid. Thin sections, made with the microtomes of v. Ardenne-Westmeyer and Niklowitz, were contrasted according to Millonig and were then investigated in the 50 kV Zeiss electron microscope, Model D-2. The lungs of all animals were also studied with the ordinary optical microscope.

### Results

One day after the start of the experiment we were already able to notice very tiny hemorrhages in some of the animal lungs. These hemorrhages became more frequent and larger as the experiment continued (Figure 1) and in the end they extended to an entire lung lobe, especially in those animals which had not been given any cumarin along with food during one day in the course of our experiment. Mucosa hemorrhages in the upper respiratory passages in that case are likewise quite extensive but they are not to be found occasionally, even though we may have lung hemorrhages. The blow against the neck leads to hemorrhage foci in the lungs already in untreated controls; this is due either to blood aspiration from the nose and throat region or it is due to central regulatory disturbances; thus we see that this blow on the neck interferes with a more detailed and accurate analysis. We therefore also used anesthesia and narcosis methods which we described earlier.

Submicroscopically, we can recognize larger bubbles in the cytoplasm of the endothelial cells quite frequently (Figure 2a). They are mostly optically empty but occasionally they contain finely-granular or homogeneous material. These vacuoles bulge forward, here and there, into the internal endothelial surface or they sit on top of a thin cytoplasm stem rather rarely, so that they protrude for some distance into the capillary lumen. On cross-sections they are situated seemingly quite freely [suspended] in the lumen [inside diameter] (Figure 2b). On the outside of the endothelium we have circumscribed elevations of the cell from the basal membrane (Figure 2c) which can be interpreted as ruptured bubbles. Such areas are frequently delimited although incompletely, by narrow endothelial protrusions (Figure 2c). In addition, cell islands occasionally directly adjoin the bubble-shaped areas. Such elevations are not as pronounced in other cases. In that case they impress us as small subendothelial vacuoles or as definitely brighter spots between the endothelium and the basal membrane, in the form of electron-optically empty, slit-shaped [slit-shaped] areas which can be identified over shorter or longer stretches, such as we find them to a very small degree already orthologically. Perhaps the previously described larger elevations might develop from such changes likewise. The pericapillary space can be swollen locally in a spindle-shaped form or it can be swollen over longer distances.

Another although very rare feature here is represented by the double endothelium cell layer (Figure 3). In between we have a mostly narrow electron-optically empty area which in some cases might be filled with flaky material. This phenomenon can be explained if we visualize laterally quite extensive bubbles which blend into each other [which are fused together] in the endothelium cell, such as they are indeed encountered occasionally. We are thus dealing with division of an endothelium cell which is simulated by an uncommonly large bubble. Another interpretation possibility here can be derived from the observation that a few blood capillaries, here and there, reveal two cell layers which are closely together, one on top of the other; this phenomenon would appear to be produced by cell protrusions and by overlapping endothelial cells; likewise, it is not entirely impossible that we might have two endothelial cell layers here which might be the result of a genuine longitudinal division. A spatial separation of these cells or cell parts is bound to result in the same picture we described above. Indeed, we can find "bubbles" in the immediate vicinity of the cell boundaries; they run into one such "vacuole" in one particular place and they leave it again in another place.

It is obvious to relate the increased capillary permeability with these bubbles which can be encountered in every stage of the experiment. Of course, the individual animals and the various capillary stretches react in different degrees of intensity. As regards the cells which leave the blood stream, we might point out first of all the rather rare occurrence of smaller hole formations in the endothelial union [endothelium] which are probably due to the fact that neighboring endothelial cells move apart [move away from each other]. Occasionally we can find a diapedesis of blood cells. In the case of leukocyte emigration we were able to recognize only one hole formation in the endothelium and it shows that the leukocyte squeezed through this hole. There were no endothelium protuberances here which might have isolated the penetrating shell from the capillary lumen. Of course, the gap which develops in the vascular wall is almost blocked by the penetrating cell. After the cell has moved out, we again get a compact endothelium layer [coat]. As a result we very frequently encounter blood cells of the red and somewhat more rarely of the white series in an extra-capillary position, without any gaps developing in the vascular wall. We might also emphasize here the rather closely adjoining deposit of erythrocytes and leukocytes against the internal endothelium cell membranes. We are dealing here with what has been called "sticking" (Florey, 1958). This is based on the increased stickiness of the two participating cells or only of one of these cells and this frequently introduces or initiates the diapedesis.

According to our findings we must assume that regressive changes in the end can lead to greater connections or bonds between the capillaries and the alveolus and this, again, makes a hemorrhage possible. When the endothelium bridge, which points toward the lumen and which has been preserved in the area of the bubble-like elevations, breaks or when a flat endothelium elevation develops in the endothelial union from the formation of a gap, then direct contact results between the content of the capillaries and the basal membrane (Figure 4). This direct contact is brought about

by a dissolution of the endothelial cell which, as a rule, involves a very heavily edematously swollen cell whose cell membrane tears or rips into the later stages. Along such defects we can find bubble-like cell segments freely in the lumen and we can interpret them as remnants of the cell. The epithelial cells of the lung alveolus can also reveal such high-grade cell edemas that they will be just about electron-optically empty [that they will just about look empty under the electron microscope]. It is extremely difficult to establish any local swellings in the basal membrane. All of these changes are suitable for the preparation of a circumscribed, larger connection between the alveolus and the capillaries. It is extremely difficult to establish these changes because it is very difficult to orient oneself in the lungs which have been altered to a very high degree. But we were convinced by one defect in those area we found isolated vacuoles and which revealed basal membrane portions which ended blind along the edge, without any bending of the capillary wall or of the epithelial cells being recognizable here according to the manner of the Kohn pores (Boatman and Martin, 1963). By the way, "capillary wall breaks" are supposed to be identifiable already histologically (Steiniger, 1952). Apart from the possibility mentioned here, we might point out the necroses of the lung parenchyma which in the later stages lead to the development of a rubble field zone but which in the beginning frequently affect the pericapillary lung portions, while the blood capillaries are still quite extensively preserved. It is understandable that such necroses can cause hemorrhages when they spill over into the blood capillaries.

The further findings on the endothelial cells involve a focus-shaped swelling of the mitochondria, the formation of highly electron-proof cytoplasm inclusions, fat deposits, and, likewise only occasionally, osmophile homogeneous or finely granular cytoplasm thickenings. The capillary lumen appears to be expanded particularly at the beginning of the experiment. The vascular wall rarely reveals individual or bunched, adjoining inward-folded areas which also include the adjoining epithelial cells of the alveolus and which have been interpreted as a slackening of the wall in combination with vascular collapse (Pak Poy and Robertson, 1959).

The epithelial cells of the alveolar walls reveal not only edematous swellings but also vacuole formations, layered lamella systems, cytosomas and inclusions which can be found particularly in the cross-section of large epithelial cells (Type II of Policard, et al, 1955). They are partly granular and very little electron-proof but in other cases they are particularly compact. As regards their osmophilia and -- to a lesser extent -- also as regards their shape, they are similar to the structures of the Ratron substance which could be placed on a blind [screen, diaphragm] without any further treatment and which could thus be inspected under the microscope. But before we equate these two structures, we must take into consideration the occurrence of such inclusions in untreated controls. We might also mention here that the ergastoplasm frequently is very well developed and that we can occasionally recognize an osmophile basic plasm thickening.

In the alveoli we can find many blood cells, crystal formations, which we do not want to discuss in greater detail here, as well as granular masses of different electron-optical density. In addition to some very loosely layered portions (Figure 5), which are somewhat like blood plasma with respect to their morphological nature, we can also find very dense, dark-looking areas in which we can locate occasionally isolated cell portions, such as a rather astonishingly well-preserved mitochondrion. Direct transitions of cell parts into this alveolar content clearly indicate that it [this content] is partly derived from cell necroses. It is impossible to establish morphologically whether the cumarin derivative or its metabolism products are deposited in the alveolus. A particularly dense [compact] content of alveoli would also appear to be derived from hemolyzed erythrocytes. In the alveoli we have large phagocytizing cells in which we can recognize inclusions which, at first sight, look rather much like the content of the alveoli whereas essentially much more compact or denser areas would indicate an obviously increased concentration of this substance (Figure 5). Pictures of erythrocyte phagocytosis are also very impressive. Leukocytes are found very rarely. They, too, reveal inclusions and a much more condensed basic plasma. Finally we can find electron-proof inclusions also freely in the alveolus. Such structures are expelled from [by] the epithelial cells, as was described earlier by Bargmann and Knoop (1956). In one rat we were able to establish electron-microscopically so-called thick-walled and thin-walled pneumocysts whose cyst membrane frequently revealed a coating of finely-tubular and roundish little bodies (Figure 6). (The author wishes to thank Prof Dr G. Pliess, Pathological Institute of the University of Hamburg, for his advice.)

The interstitial cells reveal changes similar to those found in the epithelia. In the alveolar septum we can find inflammation cells but there are very many instances in which they do not turn up. As we can see from our optical microscope investigations, we get alveolar septa, which are condensed in an inflamed, focus-shaped manner, already in untreated control animals and in rats with intramuscular terpentine oil granulomas.

#### Discussion

The administration of the cumarin derivatives leads to a series of changes which can be interpreted by means of comparison with the findings in other, defined pathological processes. Here we might mention above all the occurrence of numerous vacuoles in the cytoplasm. The enlargement of the small bubbles of the endothelia has been observed in various edemas (Meessen and Schulz, 1957; Schulz, 1957; Kisch, 1958; Schulz, 1959; Magnus, Scheunemann and Schulz, 1964; Fuchs, 1963, 1964). Here we might also particularly point out a communication from Gieseking (1959) relating to rats in which an intratracheal or intraperitoneal injection of adrenalin and histamine produced a transsudation of blood liquid into the alveolar lumen. Here again large vacuoles were observed in the endothelial cytoplasm which burst outward in various places. The liquid, which accumulated in the vacuoles, pours outward, into the interstitial space between the endothelial layer and the covering cell layer [cortical cell layer]. In this connection, the transsudate can easily lift the basal membrane off the

endothelium, after passage of the endothelial cell, and then it can spread underneath. Magnus, Scheunemann and Schulz (1964) on the other hand interpret the increased vesiculation in the blood-air tract, in the case of the Trenimon edema of the rabbit, as an active adaptation process of the cell. The liquid now flows into the cytoplasm and into the extracellular spaces in an increased quantity because of a toxically produced capillary endothelium damage; this increased liquid influx is transported, through the vacuoles of the endothelial and epithelial cells in an active fashion, into the capillaries, or it is transported from the alveolus into the intercellular space and thus into the lymphatic system. But the authors believe it possible that the little bubbles in the endothelium migrate not only to the capillary lumen but also in the opposite direction. The fact that an increase in capillary permeability can be observed after cumarin therapy emerged particularly impressively in patients with moist heart decompensation or pronounced lymphatic edema, patients, in other words, whose edemas increase after cumarin therapy (Perlick, 1959). Kuschinsky and Ludwig (1950) observed increased capillary permeability already one hour after the administration of dicumarol but in most cases this capillary permeability was no longer increased after the 11th hour. Our findings do not permit a similar delineation. Neumayr and Schmid (1948), in connection with cumarin action, likewise distinguish a prothrombin-reducing component in addition to a component which increases the capillary permeability. Under the optical microscope it was also possible to find not only hemorrhages but also edemas after major dicumarol administration (bibliography in Halse, 1950). It is obvious that the loosened structural bond of the capillary wall increases its fragility (Martin, 1960; Beller, 1960).

The focus-shaped dissolution of the capillary wall reveals a few parallels to the changes in experimental lung edema (Schulz, 1957; 1959). Large endothelial bubbles are formed here from the endothelia and the basal membrane, which is exposed toward the lumen, swells edematously, just like the epithelial cells; its cell membrane, which is turned toward the alveolus, reveals tears (Schulz, 1957, 1959). The continuing capillary wall dissolution was emphasized by Kisch (1960) in the case of the supra-*renin*-produced lung edema of the rabbit and the pneumonia in rabbits and dogs occurring after the i.v. administration of croton oil emulsion.

The tissue necroses of the lungs are comparable to the "cumarin necroses" -- a rather undesirable consequence of this therapy. They have been described in about 1-3.5% of the patients treated (Leypold and Carniol, 1961; Beller, 1960); 3-8 days after the start of treatment we first of all have pains and later on we have erythema-like reddening of the skin, finally we have hematomas and then we have necroses (Leypold and Carniol, 1960; Adam, 1960; Beller, 1961); a wide variety of skin regions are affected here but this also applies to some of the more deeper-situated tissue portions (Lieb, 1964). As far as the cause is concerned we might think here of a local Schwartzman-Sanarelli phenomenon (Beller, 1960; Lieb, 1964).

The previously-mentioned interstitial pneumonias cannot readily be related causally with the administration of cumarin because they occur, to

some extent, also in the controls (Pliess, 1964; my own observations). We cannot yet determine whether we are dealing with an accidental finding in the case of the tapestry-like pneumocystosis which we observed or whether coumarin treatment propagates pneumocystosis; this is particularly true since pneumocystoses occur without any pathological significance in various domestic and wild animals (quoted from Jirovec, 1957, 1960). Experimental pneumocystosis can be produced through a resistance loss in the experimental animals (Beller, 1955, 1956; Pliess and Trodo, 1958; Hammerl, 1960 (bibl.); Seifert and Pliess 1960; Pliess, 1964).

This leaves us with one question to be answered: to what extent are the structural changes described in this article a direct result of coumarin? Or are these structural changes connected with the inflammatory reaction of the septa. We therefore consider the influence of the coumarin preparation to be very important because the changes can be established also in places without inflammation and because they cannot be found in the case of the control animals.

In conclusion we must stress that we tried to achieve toxic effects in connection with the preparation we used because this particular preparation is used in rodent control. But the so-called coumarin necroses might make it obvious to assume that the alterations which we observed can appear also in combination with complications after the use of other coumarin preparations.

#### Summary

The changes occurring in the rat lung after administration of the coumarin derivative are examined under the electron microscope here. This produces findings pointing to increased capillary permeability. Hemorrhages under the tissue of the lung parenchyma are produced not only by a haemorrhagia per diapedesin but also by local vascular wall defects and parenchyma necroses which, in advanced experimental stages, are very extensive and which can even be compared to the so-called coumarin necroses in pharmacotherapy.

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#### PHOTO CAPTIONS

Figure 1. Lung parenchyma hemorrhage; third day of experiments, 109th rat, hematoxylin-eosin, enlarged 220X.

Figure 2a-c. Different-sized bubbles (V) in the endothelial cytoplasm (E) (Figures a, b); in Figure b they are located freely in the lumen (L) probably due to the way in which the section was made. "Endothelial elevation" (A) rising from basal membrane (BM) (Figure c) partially delimited by an endothelial cell protrusion (\*); 2nd and 3rd days of experiment, No 851/64, 4141/63, 775/64, enlarged 18,400 X, 21,300 X, 38,400 X.

Figure 3. "Double endothelium cell layer"; M -- monocyte in capillary lumen; 3rd day of experiment, No 4142/63, 35,500 X.

Figure 4. Direct contact between capillary content and basal membrane (BM). E -- endothelial cell. The marked section [in the box] is more enlarged. Ep -- epithelial cell; L -- capillary lumen; V -- vacuole; 3rd day of experiment, No 2941/64, 39,760 X, respectively, 71,000 X.

Figure 5. Loose, flaky material inside the alveolus, intra-alveolar material; Ep -- swollen epithelial cell; Ery -- erythrocyte; L -- capillary lumen; M -- alveolar macrophage, 3rd day of experiment, No 902/64, 5,145 X.

Figure 6. So-called thin-walled and thick-walled pneumocysts with finely tubular and rounded bodies. L -- capillary lumen; 2nd day of experiments; No 799/64 and 811/64, 15,265 X, respectively, 31,950 X.